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S/N 09/825,765

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Yumi Yokoyama et al.

Examiner: Unknown

Serial No.: 09/825,765

Group Art Unit: 1645

Filed: April 4, 2001

Docket: 600.491US2

Title: GENETIC MODIFICATION OF ENDOSTATIN

PRELIMINARY AMENDMENT

Commissioner for Patents  
Washington, D.C. 20231

Sir:

In response to the "Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures" mailed October 29, 2001, please amend the above-identified patent application as follows:

In the Specification

Please insert the SEQUENCE LISTING into the specification.

Please make the paragraph substitutions indicated in the appendix entitled "Clean Version of Amended Specification Paragraphs". The specific changes incorporated in the substitute paragraphs are shown in the following marked-up version of the original paragraphs:

The paragraph beginning at page 2, line 25 and continuing to page 3, line 23, is amended as follows:

The present invention provides a composition having a targeting moiety specific for endothelial cells linked to an antiangiogenic moiety. A "targeting moiety" as used herein is a molecule that facilitates the interaction and/or binding of the linked antiangiogenic moiety to endothelial cells. Preferably, the targeting moiety and the antiangiogenic moiety are polypeptides or peptides, which, when linked form a chimeric polypeptide. The term "chimeric polypeptide" refers to a protein that includes amino acid sequences or segments that are positioned or linked in a manner which does not normally occur in the native genome of a species. More preferably, the targeting moiety is a polypeptide or peptide derived from a particular extracellular matrix (ECM) polypeptide, such as human fibronectin. Fibronectin contains the tripeptide RGD (Arg-Gly-Asp), which is also found within most major types of

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matrix proteins. A "peptide" as used herein has fewer than 20 residues, e.g., fewer than 10 or fewer than 5 residues. Preferred targeting moieties bind to  $\alpha_v\beta_3/\alpha_v\beta_5$  integrins, e.g., RGD is a motif found in molecules that bind  $\alpha_v\beta_3$ , and/or to  $\alpha_5$  and/or  $\alpha_v$  integrins, as endostatin may interact with  $\alpha_5$  and/or  $\alpha_v$  integrins as well (Rehn et al., 2001). Thus, preferred peptide or polypeptide targeting moieties are derived from RGD-containing molecules such as vitronectin, osteopontin bone sialoprotein, and disintegrins, as well as other molecules which are specific for  $\alpha_v\beta_3$ , e.g., echistatin, kistrin, integrelin, tirofiban, amifiban or xemolofiban, including anti- $\alpha_v\beta_3$  antibodies. Preferred targeting moieties include but are not limited to RGD, NGR, RGDNGR (SEQ ID NO:8), NGRRGD (SEQ ID NO:9), or tandem repeats of RGD, NGR, RGDNGR (SEQ ID NO:8), or NGRRGD (SEQ ID NO:9), or any combination thereof. Preferred polypeptide or peptide targeting moieties are less than about 100, more preferably less than about 50, even more preferably less than about 10, but at least 3, residues in length, and when linked to the antiangiogenic polypeptide yield a chimeric polypeptide that inhibits proliferation and/or migration of endothelial cells, attaches to endothelial cells, and/or inhibits tumor growth, and preferably is enhanced in these properties relative to the corresponding non-chimeric antiangiogenic polypeptide. In one embodiment of the invention, the targeting moiety is RGD.

The paragraph beginning at page 6, line 7 is amended as follows:

Figure 3. Specificity of RGD-Endostatin Binding. A human melanoma cell line, WM35, expressing  $\alpha_v\beta_3$  integrin was used to determine the accessibility and specificity of RGD-endostatin mediated cell attachment. The cell attachment was examined by Olympus BX-60 microscope at 100X magnification. Representative field of attached cells are shown. A, BSA (negative control); B, Vitronectin; C, Endostatin; and D-H, RGD-Endostatin coated wells. Binding of WM35 cells to RGD-endostatin coated wells was completely blocked by preincubation with anti human  $\alpha_v\beta_3$  antibody (E); anti-HLA antibody (negative control) (F), RGDS (SEQ ID NO:11) peptide (G), or RGES (SEQ ID NO:10) peptide (negative control) (H).

The paragraph beginning at page 6, line 16 is amended as follows:

Figure 4. Binding of Integrin Positive Cells to Endostatin Coated Wells. The number of  $\alpha_v\beta_3$  positive cells attached to wells coated with different reagents was quantified by Cell Counting Kit-8 (Dojindo, Japan). Cells attached to 0.2% gelatin coated wells were used as 100%

09825765-01302

Serial Number: 09/825,765

Filing Date: April 4, 2001

Title: GENETIC MODIFICATION OF ENDOSTATIN

to calculate relative number of cells bound to RGD-endostatin coated wells. Data are expressed as a mean (columns)  $\pm$  SD (bars). Statistical significance of differences was determined using Student's t-test.  $**p < 0.01$ . RGDS is SEQ ID NO:11 and RGES is SEQ ID NO:10.

The paragraph beginning at page 39, line 18 and continuing to page 40, line 2, is amended as follows:

*Cell Attachment Assay.* One nmole/well endostatins or RGD peptide [(H)<sub>4</sub>-(G)<sub>3</sub>-R-G-D-(G)<sub>3</sub>-C] (SEQ ID NO:12), 200 nmol/well vitronectin (GIBCO BRL, Gaithersburg, MD) or 0.2% gelatin were used to coat 96 well ELISA plates, which do not allow direct cell attachment and spreading. The plates were incubated at 4°C overnight, and then blocked with 2% BSA in PBS at 37°C for 2 hours. HUVEC, MA148 (negative control), or WM35 (positive control for  $\alpha v \beta 3$  integrin expressing cell line) were harvested by the addition of 1 mM EDTA, and resuspended in EGM medium (HUVEC) or RPMI1640 medium (MA148, WM35). The cells were incubated with or without competitors (1  $\mu$ g anti- $\alpha v \beta 3$  integrin monoclonal antibody (LM609, Chemicon, Temecula, CA) or anti-HLA monoclonal antibody (negative control; G46-2.6, Pharmigen, San Diego, CA), or 25 nmole/well RGDS (SEQ ID NO:11) or RGES (SEQ ID NO:10) peptides (Sigma Chemicals, MO) for 1 hour at 37°C. Cells were added to the wells at a density of 40,000 cells/well (HUVEC and MA148) or 30,000 cells/well (WM35). After an hour-incubation at 37°C, the plates were washed for 2 times with Hank's balanced salt solution to remove unbound cells. Bound cells were detected by MTT (described later) or Cell Counting Kit-8 (Dojindo, Japan).

The paragraph beginning at page 42, line 21 and continuing to page 43, line 8 is amended as follows:

*RGD-Endostatin Increases Endothelial Cell Attachment In Vitro.* RGD-peptide is well known for its binding to integrins on the surface of endothelial cells. To determine whether the addition of a RGD-motif to endostatin can enhance the binding of endostatin to endothelial cells, cell attachment assays were performed. As a positive control, 0.2% gelatin coated wells were used. HUVEC cells attached to gelatin-coated wells were used as the standard for complete (100%) binding to calculate relative efficiency of endostatin mediated cell attachment (Figure 2A). BSA blocked wells were used as negative controls. About 30% of HUVEC bound to BSA

09/825,765-01702

coated wells. In this assay system, regular (unmodified) endostatin coated wells showed about 60% cell attachment, which was further increased by the RGD-modification (Figure 2A). RGD-endostatin ( $p < 0.05$ ) and endostatin-RGD ( $p < 0.01$ ) showed about 80% cell attachment.

Parallel experiments with RGD-containing synthetic peptide  $[(H)_4-(G)_3-R-G-D-(G)_3-C]$  (SEQ ID NO:12) showed similar binding of HUVEC. Under these experimental conditions, a preparation of recombinant murine angiostatin (kringle 1-4, expressed in yeast) did not result in endothelial cell attachment (Figure 2A). Cell attachment studies were repeated using human microvascular endothelial cells (MVEC) and bovine adrenal gland capillary endothelial cells (BCE). These studies showed a profile similar to results obtained with HUVEC (data not included).

The paragraph beginning at page 43, line 26 and continuing to page 44, line 12 is amended as follows:

The human melanoma cell line MW35, which expresses higher levels of  $\alpha v \beta 3$  integrin, was used to confirm whether the binding of the RGD moiety in endostatin is available to specifically interact with  $\alpha v \beta 3$  integrin. Representative photomicrographs of cell attachment assays are shown in Figure 3. Unlike HUVEC, MW35 cells did not bind to endostatin by itself (18%, which is similar to BSA blocked control wells) (Figures 3A, 3C, and 4). However, MW35 cells specifically attached to RGD-endostatin coated wells (60%) (Figures 3D and 4). In order to determine whether the increased binding of RGD-endostatin was indeed specific, two methods were used. In one experiment, a monoclonal antibody to anti- $\alpha v \beta 3$  integrin was used. As a control, isotype matched mouse IgG was used at a similar concentration. Preincubation of MW35 cells with the anti- $\alpha v \beta 3$  integrin antibody completely blocked cell attachment to RGD-endostatin (Figures 3E and 4). In contrast, the control antibody did not prevent MW35 cells from binding to RGD-endostatin coated wells (Figures 3F and 4). In a second series of experiment, synthetic peptides were used as competitive inhibitors. Inclusion of RGDS (SEQ ID NO:11) peptide in the medium completely prevented attachment of WM35 cells (Figures 3G and 4), whereas a control peptide, RGES (SEQ ID NO:10), did not affect MW35 cells from attaching to RGD-endostatin coated wells (Figures 3H and 4).

09/825,765-011302

Serial Number: 09/825,765

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Title: GENETIC MODIFICATION OF ENDOSTATIN

The paragraph beginning at page 45, line 15 is amended as follows:

To confirm whether the RGD moiety itself inhibited migration of endothelial cells, CGGGRGD (SEQ ID NO:13) was chemically linked to bovine serum albumin (RGD-BSA) using a heterobifunctional cross-linking reagent, N-succinimidyl 3-[2-pyridyldithio]propionate (SPDP, Pierce Chemicals, Rockford, IL). Accessibility of RGD in the BSA conjugate was validated in cell attachment assays (data not shown). Based on molecular weight shift in SDS-PAGE, an average of 5 RGD moieties were introduced per mole of BSA. RGD-BSA did not inhibit migration of HUVEC at equimolar concentration (10-50 nM concentration, data not shown).

The paragraph beginning at page 49, line 20 is amended as follows:

Binding to endothelial cells can potentially limit the bioavailability of endostatins when administered systemically. It is interesting to note that even native endostatin showed significant binding to endothelial cells. Angiostatin, another potent antiangiogenic molecule, did not favor endothelial cell attachment. Since endostatin was derived from an ECM component (collagen type XVIII), it may interact with hitherto unidentified cell surface components on the endothelial cells. The primary sequence of endostatin has no RGD motif. However a reverse sequence, DGR, is located at position 104-106 of mouse, but not human, endostatin. DGR does not bind to  $\alpha_v\beta_3$  integrin. But mouse and human endostatin have RGAD (SEQ ID NO:14), which is not expected to bind  $\alpha_v\beta_3/\alpha_v\beta_5$  integrins. Identifying the cell surface molecule recognizing endostatin is therefore important to understand the mechanism of action of endostatin.

The paragraph beginning at page 50, line 1 is amended as follows:

The addition of the RGD-sequence to endostatin significantly improved endothelial cell binding. Moreover, the increased binding was completely blocked by adding a synthetic peptide RGDS (SEQ ID NO:11) or anti- $\alpha_v\beta_3$  integrin antibody but not by RGEs (SEQ ID NO:10) peptide or a control antibody. Competitive inhibition studies using RGD-peptide did not affect the basal levels of endothelial cell attachment to endostatin-coated plates. These results indicate that RGD-modified endostatins can bind to endothelial cells both via  $\alpha_v\beta_3$  integrin and by a second, unknown target molecule. Binding of endostatin to endothelial cells appears to be

09/825,765-011702

specific since under similar conditions, endostatin did not bind significantly to an epithelial tumor cell line, which is also used in tumor growth inhibition studies.

### **In the Claims**

Please substitute the claim set in the appendix entitled "Clean Version of Pending Claims" for the previously pending claim set. Specific amendments to individual claims are detailed in the following marked up set of claims.

Please amend the claims as follows:

3. (Amended) The composition of claim 2 wherein the targeting moiety comprises RGD, NGR, RGDNGR (SEQ ID NO:8), or NGRRGD (SEQ ID NO:9).
31. (Amended) The method of claim 18 or 21 wherein the targeting moiety is RGD, NGR, RGDNGR (SEQ ID NO:8), or NGRRGD (SEQ ID NO:9).

### **Remarks**

Claims 3 and 31 are amended. Claims 1-26 are pending.

This Preliminary Amendment and the above-referenced SEQUENCE LISTING are filed to conform the above-referenced application to the requirements of 37 C.F.R. §§ 1.821 - 1.825. To conform the above-referenced application to the requirements of 37 C.F.R. §§ 1.821 through 1.825, a paper copy of a Sequence Listing is submitted herewith. The paper copy of the Sequence Listing in this application is identical to the computer readable form of the Sequence Listing filed in application Serial No. 09/825,129, filed April 03, 2001. In accordance with 37 C.F.R. § 1.821(e), please use the computer readable form filed on November 26, 2001 in application Serial No. 09/825,129 as the computer readable form for the instant application.

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Serial Number: 09/825,765

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Title: GENETIC MODIFICATION OF ENDOSTATIN


The amendments to the specification and claims are made to conform the above-referenced application to the requirements of 37 C.F.R. § 1.821(d).

Respectfully submitted,

YUMI YOKOYAMA ET AL.

By their Representatives,

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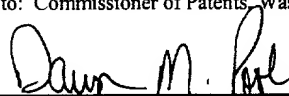
Date December 12, 2001By   
Janet E. Embretson  
Reg. No. 39,665

CERTIFICATE UNDER 37 CFR 1.8: The undersigned hereby certifies that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail, in an envelope addressed to: Commissioner of Patents, Washington, D.C. 20231, on this 12th day of December, 2001.

Name

Dawn M. Poole

Signature



09/825,765-011202



Docket No. 00600.491US2  
WD # 413855.wpd

U of M Docket No. Z00094

**Clean Version of Pending Claims**

**GENETIC MODIFICATION OF ENDOSTATIN**

Applicant: Yumi Yokoyama et al.

Serial No.: 09/825,765

1. A composition comprising: a chimeric polypeptide comprising a peptide or polypeptide targeting moiety specific for endothelial cells linked to an antiangiogenic polypeptide.
2. The composition of claim 1 wherein the targeting moiety binds to integrin on endothelial cells.
3. (Amended) The composition of claim 2 wherein the targeting moiety comprises RGD, NGR, RGDNGR (SEQ ID NO:8), or NGRRGD (SEQ ID NO:9).
4. The composition of claim 2 wherein the targeting moiety binds to  $\alpha_v\beta_3/\alpha_v\beta_5$  integrins.
5. The composition of claim 1 wherein the targeting moiety and the anti-angiogenic polypeptide are linked via a peptide bond.
6. The composition of claim 1 wherein the targeting moiety is linked to the amino terminus of the anti-angiogenic polypeptide.
7. The composition of claim 1 wherein the targeting moiety is linked to the carboxy terminus of the antiangiogenic polypeptide.
8. The composition of claim 1 wherein the antiangiogenic polypeptide is endostatin.

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9. The composition of claim 8 wherein the amino acid at position 125 in endostatin is not proline.
10. The composition of claim 9 wherein the amino acid at position 125 is alanine, valine, leucine, isoleucine or methionine.
11. The composition of claim 1 wherein the antiangiogenic polypeptide is angiostatin.
12. The composition of claim 1 wherein the antiangiostatic polypeptide is kringle 5 of plasminogen, angiostatin (kringle 1-4 of plasminogen), tumstatin, canstatin, anti-thrombin fragment or retinal pigment derived factor.
13. The composition of claim 1 further comprising a pharmaceutically acceptable diluent.
14. The composition of claim 8 wherein the targeting moiety is RGD.
15. A sustained release dosage form comprising the composition of claim 1.
16. The sustained release dosage form of claim 15 which comprises alginate beads.
17. A host cell transformed with recombinant DNA encoding a chimeric polypeptide comprising a peptide or polypeptide targeting moiety specific for endothelial cells linked to an antiangiogenic polypeptide.
18. A method to inhibit or prevent undesirable endothelial cell proliferation or migration, comprising: contacting a mammalian endothelial cell with an amount of the composition of claim 1 effective to inhibit or prevent undesirable endothelial cell proliferation or migration.

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19. The method of claim 18 wherein the mammalian cell is a human cell.
20. The method of claim 18 wherein the composition comprises a RGD-containing peptide linked to endostatin.
21. A therapeutic method comprising: administering to a mammal having a condition characterized by undesirable endothelial cell proliferation or migration, a dosage from comprising an effective amount of the composition of claim 1.
22. The method of claim 21 wherein the condition is cancer, diabetic retinopathy, macular degeneration, or restenosis.
23. The method of claim 21 wherein the condition is colon cancer.
24. The method of claim 21 wherein the condition is ovarian cancer.
25. The method of claim 21 wherein the dosage form is a sustained release dosage form.
26. The method of claim 25 wherein the sustained release dosage form comprises alginate.
27. The method of claim 18 or 21 wherein the antiangiogenic polypeptide in the composition is kringle 5 of plasminogen, angiostatin (kringle 1-4 of plasminogen), tumstatin, canstatin, anti-thrombin fragment or retinal pigment derived factor.
28. The method of claim 18 or 21 wherein the antiangiostatic polypeptide in the composition is endostatin.

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29. The method of claim 28 wherein amino acid at position 125 of endostatin is not a proline.
30. The method of claim 29 wherein the amino acid at position 125 is alanine, valine, leucine, isoleucine or methionine.
31. (Amended) The method of claim 18 or 21 wherein the targeting moiety is RGD, NGR, RGDNGR (SEQ ID NO:8), or NGRRGD (SEQ ID NO:9).

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**Clean Version of Specification Paragraphs**

**GENETIC MODIFICATION OF ENDOSTATIN**

Applicant: Yumi Yokoyama et al.

Serial No.: 09/825,765

The paragraph beginning at page 2, line 25 and continuing to page 3, line 23, is amended as follows:

The present invention provides a composition having a targeting moiety specific for endothelial cells linked to an antiangiogenic moiety. A "targeting moiety" as used herein is a molecule that facilitates the interaction and/or binding of the linked antiangiogenic moiety to endothelial cells. Preferably, the targeting moiety and the antiangiogenic moiety are polypeptides or peptides, which, when linked form a chimeric polypeptide. The term "chimeric polypeptide" refers to a protein that includes amino acid sequences or segments that are positioned or linked in a manner which does not normally occur in the native genome of a species. More preferably, the targeting moiety is a polypeptide or peptide derived from a particular extracellular matrix (ECM) polypeptide, such as human fibronectin. Fibronectin contains the tripeptide RGD (Arg-Gly-Asp), which is also found within most major types of matrix proteins. A "peptide" as used herein has fewer than 20 residues, e.g., fewer than 10 or fewer than 5 residues. Preferred targeting moieties bind to  $\alpha_v\beta_3/\alpha_v\beta_5$  integrins, e.g., RGD is a motif found in molecules that bind  $\alpha_v\beta_3$ , and/or to  $\alpha_5$  and/or  $\alpha_v$  integrins, as endostatin may interact with  $\alpha_5$  and/or  $\alpha_v$  integrins as well (Rehn et al., 2001). Thus, preferred peptide or polypeptide targeting moieties are derived from RGD-containing molecules such as vitronectin, osteopontin bone sialoprotein, and disintegrins, as well as other molecules which are specific for  $\alpha_v\beta_3$ , e.g., echistatin, kistrin, integrelin, tirofiban, amifiban or xemolofiban, including anti- $\alpha_v\beta_3$  antibodies. Preferred targeting moieties include but are not limited to RGD, NGR, RGDNGR (SEQ ID NO:8), NGRRGD (SEQ ID NO:9), or tandem repeats of RGD, NGR, RGDNGR (SEQ ID NO:8), or NGRRGD (SEQ ID NO:9), or any combination thereof. Preferred polypeptide or peptide targeting moieties are less than about 100, more preferably less than about 50, even more preferably less than about 10, but at least 3, residues in length, and when linked to the

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antiangiogenic polypeptide yield a chimeric polypeptide that inhibits proliferation and/or migration of endothelial cells, attaches to endothelial cells, and/or inhibits tumor growth, and preferably is enhanced in these properties relative to the corresponding non-chimeric antiangiogenic polypeptide. In one embodiment of the invention, the targeting moiety is RGD.

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The paragraph beginning at page 6, line 7 is amended as follows:

Figure 3. Specificity of RGD-Endostatin Binding. A human melanoma cell line, WM35, expressing  $\alpha v \beta 3$  integrin was used to determine the accessibility and specificity of RGD-endostatin mediated cell attachment. The cell attachment was examined by Olympus BX-60 microscope at 100X magnification. Representative field of attached cells are shown. A, BSA (negative control); B, Vitronectin; C, Endostatin; and D-H, RGD-Endostatin coated wells. Binding of WM35 cells to RGD-endostatin coated wells was completely blocked by preincubation with anti human  $\alpha v \beta 3$  antibody (E); anti-HLA antibody (negative control) (F), RGDS (SEQ ID NO:11) peptide (G), or RGES (SEQ ID NO:10) peptide (negative control) (H).

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The paragraph beginning at page 6, line 16 is amended as follows:

Figure 4. Binding of Integrin Positive Cells to Endostatin Coated Wells. The number of  $\alpha v\beta 3$  positive cells attached to wells coated with different reagents was quantified by Cell Counting Kit-8 (Dojindo, Japan). Cells attached to 0.2% gelatin coated wells were used as 100% to calculate relative number of cells bound to RGD-endostatin coated wells. Data are expressed as a mean (columns)  $\pm$  SD (bars). Statistical significance of differences was determined using Student's t-test. \*\* $p < 0.01$ . RGDS is SEQ ID NO:11 and RGES is SEQ ID NO:10.

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The paragraph beginning at page 39, line 18 and continuing to page 40, line 2, is amended as follows:

*Cell Attachment Assay.* One nmole/well endostatins or RGD peptide [(H)<sub>4</sub>-(G)<sub>3</sub>-R-G-D-(G)<sub>3</sub>-C] (SEQ ID NO:12), 200 nmol/well vitronectin (GIBCO BRL, Gaithersburg, MD) or 0.2% gelatin were used to coat 96 well ELISA plates, which do not allow direct cell attachment and spreading. The plates were incubated at 4°C overnight, and then blocked with 2% BSA in PBS at 37°C for 2 hours. HUVEC, MA148 (negative control), or WM35 (positive control for αvβ3 integrin expressing cell line) were harvested by the addition of 1 mM EDTA, and resuspended in EGM medium (HUVEC) or RPMI1640 medium (MA148, WM35). The cells were incubated with or without competitors (1 μg anti-αvβ3 integrin monoclonal antibody (LM609, Chemicon, Temecula, CA) or anti-HLA monoclonal antibody (negative control; G46-2.6, Pharmigen, San Diego, CA), or 25 nmole/well RGDS (SEQ ID NO:11) or RGES (SEQ ID NO:10) peptides (Sigma Chemicals, MO) for 1 hour at 37°C. Cells were added to the wells at a density of 40,000 cells/well (HUVEC and MA148) or 30,000 cells/well (WM35). After an hour-incubation at 37°C, the plates were washed for 2 times with Hank's balanced salt solution to remove unbound cells. Bound cells were detected by MTT (described later) or Cell Counting Kit-8 (Dojindo, Japan).

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The paragraph beginning at page 42, line 21 and continuing to page 43, line 8 is amended as follows:

*RGD-Endostatin Increases Endothelial Cell Attachment In Vitro.* RGD-peptide is well known for its binding to integrins on the surface of endothelial cells. To determine whether the addition of a RGD-motif to endostatin can enhance the binding of endostatin to endothelial cells, cell attachment assays were performed. As a positive control, 0.2% gelatin coated wells were used. HUVEC cells attached to gelatin-coated wells were used as the standard for complete (100%) binding to calculate relative efficiency of endostatin mediated cell attachment (Figure 2A). BSA blocked wells were used as negative controls. About 30% of HUVEC bound to BSA coated wells. In this assay system, regular (unmodified) endostatin coated wells showed about 60% cell attachment, which was further increased by the RGD-modification (Figure 2A). RGD-endostatin ( $p < 0.05$ ) and endostatin-RGD ( $p < 0.01$ ) showed about 80% cell attachment. Parallel experiments with RGD-containing synthetic peptide  $[(H)_4-(G)_3-R-G-D-(G)_3-C]$  (SEQ ID NO:12) showed similar binding of HUVEC. Under these experimental conditions, a preparation of recombinant murine angiostatin (kringle 1-4, expressed in yeast) did not result in endothelial cell attachment (Figure 2A). Cell attachment studies were repeated using human microvascular endothelial cells (MVEC) and bovine adrenal gland capillary endothelial cells (BCE). These studies showed a profile similar to results obtained with HUVEC (data not included).

The paragraph beginning at page 43, line 26 and continuing to page 44, line 12 is amended as follows:

The human melanoma cell line MW35, which expresses higher levels of  $\alpha v \beta 3$  integrin, was used to confirm whether the binding of the RGD moiety in endostatin is available to specifically interact with  $\alpha v \beta 3$  integrin. Representative photomicrographs of cell attachment assays are shown in Figure 3. Unlike HUVEC, MW35 cells did not bind to endostatin by itself (18%, which is similar to BSA blocked control wells) (Figures 3A, 3C, and 4). However, MW35 cells specifically attached to RGD-endostatin coated wells (60%) (Figures 3D and 4). In order to determine whether the increased binding of RGD-endostatin was indeed specific, two methods were used. In one experiment, a monoclonal antibody to anti- $\alpha v \beta 3$  integrin was used. As a control, isotype matched mouse IgG was used at a similar concentration. Preincubation of MW35 cells with the anti- $\alpha v \beta 3$  integrin antibody completely blocked cell attachment to RGD-endostatin (Figures 3E and 4). In contrast, the control antibody did not prevent MW35 cells from binding to RGD-endostatin coated wells (Figures 3F and 4). In a second series of experiment, synthetic peptides were used as competitive inhibitors. Inclusion of RGDS (SEQ ID NO:11) peptide in the medium completely prevented attachment of WM35 cells (Figures 3G and 4), whereas a control peptide, RGES (SEQ ID NO:10), did not affect MW35 cells from attaching to RGD-endostatin coated wells (Figures 3H and 4).

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The paragraph beginning at page 45, line 15 is amended as follows:

To confirm whether the RGD moiety itself inhibited migration of endothelial cells, CGGGRGD (SEQ ID NO:13) was chemically linked to bovine serum albumin (RGD-BSA) using a heterobifunctional cross-linking reagent, N-succinimidyl 3-[2-pyridyldithio]propionate (SPDP, Pierce Chemicals, Rockford, IL). Accessibility of RGD in the BSA conjugate was validated in cell attachment assays (data not shown). Based on molecular weight shift in SDS-PAGE, an average of 5 RGD moieties were introduced per mole of BSA. RGD-BSA did not inhibit migration of HUVEC at equimolar concentration (10-50 nM concentration, data not shown).

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The paragraph beginning at page 49, line 20 is amended as follows:

Binding to endothelial cells can potentially limit the bioavailability of endostatins when administered systemically. It is interesting to note that even native endostatin showed significant binding to endothelial cells. Angiostatin, another potent antiangiogenic molecule, did not favor endothelial cell attachment. Since endostatin was derived from an ECM component (collagen type XVIII), it may interact with hitherto unidentified cell surface components on the endothelial cells. The primary sequence of endostatin has no RGD motif. However a reverse sequence, DGR, is located at position 104-106 of mouse, but not human, endostatin. DGR does not bind to  $\alpha_v\beta_3$  integrin. But mouse and human endostatin have RGAD (SEQ ID NO:14), which is not expected to bind  $\alpha_v\beta_3/\alpha_v\beta_5$  integrins. Identifying the cell surface molecule recognizing endostatin is therefore important to understand the mechanism of action of endostatin.

00600.491US2-01102

The paragraph beginning at page 50, line 1 is amended as follows:

The addition of the RGD-sequence to endostatin significantly improved endothelial cell binding. Moreover, the increased binding was completely blocked by adding a synthetic peptide RGDS (SEQ ID NO:11) or anti- $\alpha_v\beta_3$  integrin antibody but not by RGES (SEQ ID NO:10) peptide or a control antibody. Competitive inhibition studies using RGD-peptide did not affect the basal levels of endothelial cell attachment to endostatin-coated plates. These results indicate that RGD-modified endostatins can bind to endothelial cells both via  $\alpha_v\beta_3$  integrin and by a second, unknown target molecule. Binding of endostatin to endothelial cells appears to be specific since under similar conditions, endostatin did not bind significantly to an epithelial tumor cell line, which is also used in tumor growth inhibition studies.

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